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Tubagus Bahtiar Rusbana

(B7AD1309)

Laboratory of Nutrition
Department of Science of Food Function and Health
Division of Bioscience and Biotechnology for Future Bioindustries
Graduate School of Agricultural Science
Tohoku University

Supervisor
Prof. Hitoshi Shirakawa

TABLE OF CONTENTS

LIST OF PUBLICATIONS

1. **Tubagus Bahtiar Rusbana, Afifah Zahra Agista, Wahyu Dwi Saputra, Yusuke Ohsaki, Kouichi Watanabe, Ardiansyah, Slamet Budijanto, Takuya Koseki, Hisashi Aso, Michio Komai, and Hitoshi Shirakawa** (2020) Supplementation with fermented rice bran attenuates muscle atrophy in a diabetic rat model. *Nutrients* 12: 2409.

2. **Tubagus Bahtiar Rusbana, Yusuke Ohsaki, Michio Komai, and Hitoshi Shirakawa.** (2020) Effect of dietary vitamin K on cognitive impairment in senescence accelerated mouse prone 8 (SAMP8). (In preparation)

GENERAL INTRODUCTION

The ageing process is commonly defined as the accumulation of diverse deleterious changes occurring in cells and tissues with advancing age. These deteriorations process leads to a gradual decrease in physiological organs, an increased risk of many diseases, and a general decline in the capacity of the individual. This process is often severed by non-communicable diseases (NCDs) such as diabetes mellitus.

Locomotive syndrome and cognitive impairment are the main risks of ageing which could be accelerated by diabetes mellitus. The combination of ageing and NCDs leads to impaired functions and increased vulnerability to morbidity and mortality which bring a huge economic burden to society. The efforts to deal with the effects of ageing is an important strategy considering that the world is currently experiencing demographic problems.

There are some interventions and research for facing up the aging which is lied based on the theory of aging. The free radical, immunologic, inflammation, mitochondrial, and neuroendocrine theory are all theories of the aging pathway, providing useful and important insights for understanding the physiological changes and for guidance on how the treatment that could be searched and could be done.

Recent findings show that vitamin K (VK) status and rice bran supplementation influenced the health and ageing process. The high abundance of VK in the normal brain and negative correlation between ageing and VK status on cognitive impairment in elder people became the background of this study.

Fermented rice bran (FRB) as the rich functional ingredient sources, both its fraction and single compound extract, in several studies showed beneficial function for improving the metabolic syndrome, diabetes mellitus, and other lifestyle diseases by its anti-inflammation and anti-oxidative effect.

Using two models of animal, VK and FRB are expected to exhibit the nutraceuticals effect on amelioration of ageing process. Streptozotocin-induced diabetic rat which is used in this study provides the muscle atrophy condition as a sarcopenia model. FRB with its functional compounds is expected to give the improvement on muscle atrophy during diabetic complications. Senescence accelerated mouse prone 8 (SAMP8) is the mouse model for cognitive impairment which has behavioral alterations as young as 4 months old is expected to show the influence of VK concentration on the diet to cognitive impairment. We will not be able to abolish ageing, but we do expect to be able to attenuate the process and greatly ameliorate its effects (Partridge et al., 2018).

AIM OF THE STUDY

The general objective of this study is to determine the physiological effect of FRB and VK on age-related diseases in the animal models. In particular, the aims of the study are as follows:

1. To determine the effect of FRB in muscle atrophy on a streptozotocin-induced diabetic rat model.
2. To elucidate the effect of VK on cognitive impairment on SAMP8.

LITERATURE REVIEW

Muscle atrophy and cognitive impairment as the risks of ageing and non-communicable diseases (NCDs)

The increased life expectancy and the declining birth rate have rapidly escalated the aged population in the world. The global population aged 60 years or over numbered 962 million in 2017, more than twice as large as in 1980, is expected to double by 2050 to reach nearly 2.1 billion (United Nations, 2017). Accompanied the ageing population, NCDs also become the challenges that are facing the world today. The global burden disease study showed 88% of age-related diseases are NCDs with diabetes mellitus and neurological disorders are included as the top ten of age-related diseases (Chang et al., 2019). The aging population brought a huge economic burden to society, which is also increased the need for integrated care in the community instead of hospital care (Woo, 2017), because of the impaired functions and the increased vulnerability to morbidity and mortality.

Theoretically, ageing is caused by several processes which are well explained by Tosato et al. (2007), and briefly, it can be classified into two categories: stochastic and non-stochastic. The stochastic group is referred to molecular disruption due to the actions of free radicals species resulting a deterioration effect on the cell's elements, while the non-stochastic is referred to the disruption of genes with advancing the age with dwelling the milieu factors (Trevisan et al., 2019). In this research, we followed the stochastic approach, in which oxidative stress (free radical) induced inflammation caused age-related symptoms like sarcopenia and cognitive impairment. Basically, inflammation is the

mechanism of organisms to fight the invasion of microorganisms and to maintain the organs. However, when inflammation becomes prolonged, due to chronic oxidative stress in many chronic diseases including obesity, cardiovascular disease, and neuro-degenerative diseases, inflammation can lead to the accumulation of damage and promote the inflammaging (Bektas et al., 2018).

Sarcopenia and cognitive impairment are prevalent features of advanced aging. As an age-related disease, sarcopenia is highly prevalent in individuals with NCDs including diabetes mellitus and dementia (Pacifico et al., 2020). Sarcopenia is characterized by progressive and generalized loss of skeletal muscle mass and strength due to muscle atrophy which contributes to the risk of functional impairment in older people (Shaw et al., 2017). The chronic complication of diabetes mellitus, due to hyperglycaemic induced oxidative stress, could lead to muscle atrophy by the activation of the ubiquitin-proteasome system by involving several pathways and may contribute to muscle protein degradation by removing contractile proteins and organelles, resulting in the shrinkage of muscle mass and myofiber size (Bonaldo and Sandri, 2013; Roy, 2013).

Muscle atrophy and inflammation

Muscle atrophies eventuate when the protein catabolic rates exceed its anabolic level, which is can caused by various factors, such as aging, chronic diseases, nutritional deficiencies, genetics, and physical unloading owing to an injury or illness. Protein degradation leading to muscle atrophy in aging and diabetes are induced by oxidative stress caused hyperglycaemic milieu which triggering the inflammation (Meng and Yu, 2010; Moylan and Reid, 2007). The degradation of protein which caused diabetic muscle atrophy, is managed by two system: the ubiquitin-proteasomal (UPS) and autophagic–lysosomal system (Bonaldo and Sandri, 2013; Sandri, 2013; Schreiber and Peter, 2014).

The UPS is signed by the elevation of expression of F-box only protein 32/atrophy gene 1 (FBXO32/Atrogin-1) and tripartite motif-containing 63/muscle-specific RING finger protein 1 (TRIM63/MuRF1), two muscle-specific ubiquitin ligases which are managed by the Forkhead box subfamily O (FOXO) (Bodine and Baehr, 2014; Gomes et al., 2001; Sandri, 2013). The activity of UPS in diabetic circumstance is also increased by nuclear factor-kappa B (NF-κB) activation which is triggered owing to hyperglycemic-induced oxidative stress conditions (Eley and Tisdale, 2007; Roy, 2013). However, some studies showed that this mechanism is could inhibited by several natural compounds such as eicosapentaenoic acid and resveratrol (Whitehouse and Tisdale, 2003; Wyke et al., 2004).

The autophagic–lysosomal system is the auto degradation system of cell for degrading and recycling cellular elements such as organelles, cytoplasm, and protein content (Sandri, 2013). Furthermore, in diabetic circumstances, this process marked by the elevation of GABA type A receptor-associated protein-like 1

(GABARAPL1) and BCL2 interacting protein 3 like (BNIP3L) expression (O'Neill et al., 2019). The evaluation of these markers has been widely used to elucidate the possible mechanisms underlying muscle atrophy, especially in diabetic condition.

Dual fermentation rice bran and its function

Nutrient intervention to maintain muscle health is one of the strategic approaches on recovering from illness, also prevent and treat muscle weakness in the elderly (de D. Beas-Jiménez et al., 2011; Magne et al., 2013). Rice bran, a by-product of the rice milling industry, is the rich source of various nutrients and bioactive compounds especially in lipid fraction such as gamma-oryzanol, tocotrienol, tocopherol and α-sitosterol, and it derivate, ferulic acid (Alauddina et al., 2017).

The FRB that was utilized in this study, was prepared by dual fermentation of defatted rice bran using fungi and lactic acid bacteria (LAB) sequentially. Started with *Aspergillus kawachii*, the fermentation process is continued by a mixture of LAB (*Lactobacillus brevis, Lactobacillus rhamnosus, and Enterococcus faecium*). This dual-sequential fermentation improved the flavour characteristic and nutrients composition of the rice bran with higher lipid, dietary fibre, and total phenolic contents than those of native rice bran (Alauddin et al., 2016). Some studies of this dual fermentation of RB—as a prospective supplement—showed that the FRB has an ability on ameliorating certain medical state such as hypertension, inflammatory bowel disease, and metabolic syndrome (Alauddin et al., 2016; Ardiansyah et al., 2019; Islam et al., 2017; Yu et al., 2019).

Ferulic acid is suggested as major active component of this FRB since the raw material was defatted rice bran. Ferulic acid has showed the ability to improve the mitochondrial biogenesis and reducing oxidative stress, in a mouse model of vascular damage (Perez-Ternero et al., 2017). Moreover, phytosterol ferulic acid esters or gamma-oryzanol significantly inhibit iNOS expression and NO production,

concurrently inhibited IκBα degradation, resulted in the interfere of NF-κB nuclear translocation (Islam et al., 2011). Chronic administration of rice bran enzymatic extract showed the anti-inflammatory and anti-oxidative action in atherosclerosis animal model by decreased the mRNA of interleukine-6 (*Il6*) and tumor necrosis factor α (*Tnfa*) expressions (Perez-Ternero et al., 2016).

Brain inflammaging, cognitive impairment, and BDNF

Advancing age, we will meet the gradual changes in the brains and bodies. Systemic change of aging roughly including the changes in body composition, disruption of energy demand/supply, dysregulation of homeostasis system, and neurodegeneration with cognitive impairment (Bektas et al., 2018). The elevated inflammation with age is a natural response of cell senescence, but the exaggerated inflammatory due to the combination of aging and chronic inflammation by age-related diseases and infection response will contribute to the faster emerge of cognitive decline (Sartori et al., 2012). The accumulation of inflammasome in the brain will activate the double-effect of the senescence-associated secretory phenotype which triggers clearance of senescent cells by the innate immune system and generates a pro-inflammatory circumstance promoting inflammaging concurrently (Mészáros et al., 2020) that could leads to cognitive impairment.

Brain-derived neurotrophic factor (BDNF), as one of the neurotrophin, is the important factor in brain maintenance. This neurotrophin promotes the differentiation and survival of neurons and regulates the synaptogenesis and synaptic plasticity mechanisms which influences the ability on learning and memory in the adult central nervous system (Cunha, 2010). BDNF levels decline with increasing age (Erickson et al., 2010) and Palasz et al. (2020) also showed that the deflation on BDNF levels in the blood and brain was observed in patients with neurological diseases. Moreover, BDNF has the anti-inflammatory effect on reduced the levels of TNF-α and IL-6 in hyperglycaemic-induced microglia (Han et al., 2019) .

BDNF expression is regulated by activation of CREB via several pathway including phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), protein kinase A (PKA) and protein kinase C (PKC) (Amidfar et al., 2020). Regarding the correlation with VK, there is the similarity between the result of VK roles effect on stimulation of testosterone production via activation of PKA and CREB (Ito et al., 2011). The stimulation on activation of PKA and CREB in neuronal cell will enhance the production of BDNF and improve brain maintenance (Huang et al., 2015; You et al., 2019; Zhen et al., 2016), conversely, the down-regulation of this pathway resulted in the learning ability impairment (Wang et al., 2017; Zhong et al., 2018).

VK in the brain: its possibility effect and mechanism in brain maintenance

As an essential nutrient, VK should be supplied from the diet. The most widely consumed dietary form of VK is phylloquinone (vitamin K1) which is provided on green leafy vegetables (Booth, 2012), while another natural forms are menaquinones which provide in a specific food such as natto from Japan (Sato et al., 2020; Tsukamoto et al., 2000). Most of the menaquinone are synthesized by microorganisms, while specifically MK-4 is synthesized by the conversion of orally ingested VK1 or menaquinone in the major tissues (Komai and Shirakawa, 2007).

After absorption in the intestine, in contrast to vitamin K1 which is transported by triacylglycerol-rich lipoprotein and low-density lipoprotein, MK-4 was even detected in high-density lipoprotein. This is one of the reason that MK-4 may have a different distribution profile and that relatively have the large impact of menaquinones on extrahepatic tissues (Schurgers and Vermeer, 2002). MK-4 was detected in all tissues, with low levels in plasma and liver, and its levels exceeded vitamin K1 levels in the brain, pancreas, salivary gland, and sternum (Thijssen and Drittij-Reijnders, 1994). UbiA prenyltransferase domain-containing protein 1 (UBIAD1) prenyltransferase facilitates the conversion of VK1 into MK-4. The process started with cleaving the side chain of VK1 become menadione (VK3). The UBIAD1 catalyses the transfer of geranylgeranyl pyrophosphate bind to VK3 on forming MK-4 (Nakagawa et al., 2010).

Many studies reveal that MK-4 is the predominant VK form in the brain (Ferland et al., 2016; Shirakawa et al., 2014; Thijssen and Drittij-Reijnders, 1994) which directs the attention to the function of this vitamer on the brain and cognitive impairment. Furthermore, an epidemiological study has reported that higher serum

phylloquinone concentration was associated with better verbal episodic memory performances (Presse et al., 2013) and higher dietary phylloquinone intake was associated with better cognition and behaviour among older adult (Chouet et al., 2015). A cohort study of older Irish adults also showed that higher levels of dietary phylloquinone were found in those with better cognition, while those with the poorest cognition correlated with lower levels of phylloquinone and higher levels of inflammatory blood markers (Kiely et al., 2020). Another study using an animal model showed that warfarin-induced MK-4 deficiency resulted in lower locomotor activity, exploratory behaviour, and lower performances in the open field test (Tamadon-Nejad et al., 2018). These studies have revealed that VK status correlated with cognitive interference.

VK functions primarily as an enzymatic cofactor in the carboxylation of both hepatic and extra-hepatic vitamin K-dependent proteins (Schurgers et al., 2001). Some evidence that VK correlated with cognition represents the other role of vitamin K. The strong associations between brain MK-4 and sphingomyelin, sulfatides, and gangliosides expression suggest that this vitamer may play an important role in the brain (Carrié et al., 2004; Tamadon-Nejad et al., 2018). Supporting these findings, the tetrasialoganglioside GQ1b in hippocampal CA1 neurons of brain slices showed that GQ1b enhanced ATP-induced long-term potentiation which improves spatial learning and memory in rats (Jung et al., 2008; Shin et al., 2019) by regulates brain-derived neurotrophic factor (BDNF) expression (Shin et al., 2014).

Despite of the enzymatic cofactor role, our laboratory previously found that VK has shown an anti-inflammatory effect by suppressing inflammatory

cytokine levels in rodent models (Ohsaki et al., 2010; Ohsaki et al., 2006). Besides, our laboratory also found that MK-4 stimulates testosterone production in rats and testis-derived tumor cells via activation of PKA and CREB (Ito et al., 2011). Other studies showed that inhibition of this similar pathway on the hippocampus in rat models resulted in the lowering expression of BDNF (Chou et al., 2020; Zhong et al., 2018). These shreds of evidence raise the hypothesis that VK may protect the brain condition during aging by activates the PKA-CREB-BDNF pathway in the hippocampus.

Streptozotocin-diabetic and SAMP8

The STZ-induced diabetic murine model has been widely used to evaluate the effect of various both natural and artificial compounds in improving the chronic complications of diabetes (Wu and Yan, 2015). The chronic inflammation that has been implicated in this model due to hyperglycemia, hypoinsulinemia, and oxidative stress caused the change in metabolism pathway (Hirata et al., 2019; Oguntibeju, 2019; Ono et al., 2015) therefore, serves as an appropriate model to emulate muscle atrophy as a diabetic complication.

The senescence-accelerated mouse prone 8 (SAMP8) strain is a spontaneous animal model of accelerated aging. Many studies signify that SAMP8 mice correspond the behavioral and histopathological marks of Alzheimer disease (Cheng et al., 2014). These mice showed a mild prolongation time on T-maze and an impairment on Morris water maze test which observed even at 2 months of age. These results indicate that SAMP8 shows age-related deterioration of ability in learning and memory since 2 months of age (Miyamoto et al., 1986). Regarding the inflammation marker, study on the hippocampus SAMP8 at 2 and 9-month of age, as well as age-matched senescence-accelerated mouse resistant 1 (SAMR1) showed that pro-inflammatory markers of SAMP8 not only increased upon age but also higher than those in age-matched SAMR1 (Wang et al., 2015). Using these two ageing animal models, VK and FRB are expected to exhibit the nutraceuticals effect that ameliorates the ageing process.

CHAPTER 1

Supplementation with FRB attenuates muscle atrophy in a diabetic rat model

Introduction

The severe state of atrophied muscles could lead to an impaired ability to perform daily functions which increases dependency (Faulkner et al., 2007; Matsumoto et al., 2016) and promote the increased of health costs (Beaudart et al., 2014). This severe state could reached as the consequence of diabetic complication contributing to the increase of morbidity (O'Neill et al., 2019).

Nutritive intervention is one of the approaches to maintain muscle health (de D. Beas-Jiménez et al., 2011; Magne et al., 2013). In this study we use FRB as prospective intervention product. Previously it has been showed that FRB in improving some medical condition (Alauddin et al., 2016; Ardiansyah et al., 2019; Islam et al., 2017; Yu et al., 2019). Since this ability also important in preventing muscle atrophy, we hypothesized that FRB could be useful in improving diabetic induced muscle atrophy.

Chronic inflammation as pathophysiology of diabetes mellitus which resulted in muscle atrophy are induced in response to oxidative stress and inflammation (Meng and Yu, 2010; Moylan and Reid, 2007). Using STZ-induced diabetes animal model, we studied the expression of atrogenes, including Fbxo32/Atrogin1 and Trim63/Murf1, and the associated protein level to evaluate the ameliorative effects of FRB on diabetic induced muscle atrophy.

Materials and Methods

Materials and methods which were used in this study was presented in Rusbana et al. (2020). Especially for western blotting it should be noted that regarding the result of Fortes et al. (2016) we used Coomassie brilliant blue (CBB) staining for total protein quantification. Similarly, on the quantitative RT-PCR, we referred to Islam et al. (2017) on using eukaryotic elongation factor 1α1 to normalize the mRNA expression result. Briefly it is described below.

Materials

The components of the AIN-93M standard diet (Reeves et al., 1993) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan). FRB supplied by Sunstar Company (Tokyo, Japan). STZ was purchased from Wako Pure Chemical Industries. Goat anti-rat IL-1β (R&D Systems, Minneapolis, MN, USA) and rabbit anti-FBXO32/Atrogin-1, TRIM63/MuRF1 (ECM Biosciences, Versailles, KY, USA), ubiquitin, TNF-α, and NF-κB p65 and phosphorylated p65 (Cell Signaling Technology, Danvers, MA, USA) antibodies were used for measuring protein expression.

Animal

Eighteen male Sprague-Dawley rats (8 weeks old) were obtained from Japan SLC (Hamamatsu, Japan), divided into three groups (n = 6 per group), and house-controlled in individual cages (temperature: 23 ± 3°C; humidity: 50 ± 10%; and a 12/12–h light-dark cycle). After 1 week of acclimatization, two of the three groups were injected intraperitoneally with STZ (40 mg/kg body weight after being fasted for 6 h prior), as the diabetic groups included the STZ group and the FRB group, and one group—the control group—was injected with vehicle (0.05 M

citrate buffer, pH 4.5, after being fasted for 6 h prior). A dose of 40 mg/kg was deemed suitable as it sufficiently induced diabetic symptoms while maintaining stable conditions in rats. Two days post-treatment, blood was drawn from the tail vein, and the blood glucose level was measured using a glucometer. Rats with fasting blood glucose over 200 mg/dL were considered diabetic and used for the subsequent treatments. One rat was found to be unresponsive to the STZ treatment and therefore was excluded from further analysis.

The STZ group and the control group were fed the AIN93M diet (standard diet), and the FRB group was fed 10% of FRB—based on the AIN93M diet— for one month. Details of the experimental diets referred to Alauddin et al. (2016). The body weight was measured; additionally, the plasma glucose and insulin levels were analyzed using blood, which was collected from the tail vein. On the thirtieth day, the rats were euthanized after fasting for 6 h. Blood and the gastrocnemius muscles of both the hindlimbs were collected. The animal use and care protocols were reviewed and approved by the Animal Research-Animal Care Committee of Tohoku University according to the Japanese governmental legislation (2005). The approved document number of this animal experiment is 2018AgA-014.

Histological and blood analysis

The gastrocnemius muscle from the left hindlimb was isolated, fixed in 4% formaldehyde, treated with a series concentration of ethanol and toluene, embedded in paraffin, and sectioned into 5-μm slices. The paraffin-embedded sections were then stained with hematoxylin and eosin using standard techniques. Images of the myocyte cross-sectional areas of the muscle were captured using a digital camera at 200x magnification, and 100 fibers per animal (average) were

observed. The ImageJ software (https://imagej.nih.gov/ij/) was used to analyze the cross-sectional image of the myocytes. The blood for the estimation of the plasma glucose and insulin levels was drawn from the tail vein. The blood glucose level was measured via a colorimetric method using the glucose C2 test Wako kit (Wako Pure Chemical Industries, Ltd.), and the insulin level was measured using the rat insulin ELISA kit (Morinaga, Yokohama, Japan) according to the manufacturers' instructions. The blood profile was analyzed by Oriental Yeast Co. using an automatic biochemistry analyzer (Model 7180 Automatic Analyzer: Hitachi, Ltd., Tokyo, Japan).

Western blotting

The gastrocnemius muscle was homogenized in phosphate-buffered saline supplemented with a protease inhibitor cocktail (Complete protease inhibitor cocktail; Roche Applied Science, Mannheim, Germany) and a phosphatase inhibitor cocktail (PhosSTOP phosphatase inhibitor cocktail, Roche Applied Science). The concentrations of the protein lysates were measured by spectrophotometry at 565 nm using a protein assay (Bio-Rad, Hercules, CA, USA). The lysate (16 μg) was separated on a 12.5% sodium dodecyl sulphate polyacrylamide gel. The separated proteins were then transferred onto an Immobilon-P membrane (Millipore, Billerica, MA, USA). The membrane was incubated in a blocking buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20, and 5% skim milk or 3% bovine serum albumin] for 1 h. Subsequently, the membrane was incubated overnight with a blocking buffer containing primary antibodies. Primary antibodies for FBXO32/Atrogin-1 and TRIM63/MuRF1 were diluted 1:1000 in blocking buffer containing skim milk, whereas antibodies for

ubiquitin, IL-1β, TNF-α, and NF-κB p65 and phosphorylated p65 were diluted 1:1000 in blocking buffer containing bovine serum albumin. Immobilon western detection reagent (Millipore) was used with a luminescent image analyzer (LAS-4000 mini; Fujifilm, Tokyo, Japan). Oxidized protein was measured using the protein carbonyl assay kit (Abcam, Tokyo, Japan) following the manufacturers' protocol.

RNA extraction and quantitative RT-PCR

The gastrocnemius muscle sample was homogenized using ISOGEN (Nippon Gene, Co., Ltd., Tokyo, Japan) to isolate and purify the total RNA, and the quality and quantity of RNA were evaluated by measuring the absorbance at 260 nm and 280 nm on a spectrophotometer. cDNA was then synthesized using the isolated RNA as a template. Briefly, RNA was incubated with 5 μM oligo-dT primer (Hokkaido System Science Co., Sapporo, Japan) and 1 mM dNTP (GE Healthcare, Tokyo, Japan) at 65°C for 5 min. This denatured RNA mixture was then added to a solution containing RT buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 5 mM dithiothreitol], 50 U SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and 20 U RNaseOUT RNase inhibitor (Invitrogen). Reverse transcription was carried out at 50°C for 60 min to produce cDNA. Then, the quantitative RT-PCR was performed on CFX Connect Real-Time PCR Detection System (Bio-Rad) using gene-specific primers (Rusbana et al. 2020) and the TB Green Premix EX Taq (Takara Bio, Otsu, Japan).

Statistical analysis

Statistical analysis was performed using SigmaPlot version 12.5 (San Jose, CA, USA) and conducted with a significance level of $\alpha = 0.05$ ($p < 0.05$). Tukey’s

post hoc test was used to analyse the differences between the control, STZ, and FRB groups after ANOVA, additionally, the body weights data was analysed using repeated measures ANOVA.

Results and discussion

The injection of 40 mg of STZ per kg body weight succeed in making muscle atrophy symptom in our model (**Figure 1.1**) as the result of the pancreatic β cells destruction by STZ which was showed in the blood profile evaluation (**Table 1.1**). This study showed STZ-induced glucotoxicity caused the decreased in bodyweight followed by shrinkage of muscle fibers (**Figure 1.2**) and one-month FRB supplementation could reversed the loss of gastrocnemius muscle mass and significantly ameliorated rats` muscle shrinkage concurrently (**Figure 1.3**).

The investigation on mRNA expression of cell surface stimulators, inflammatory cytokines, muscle atrophy and mitophagy related genes showed that FRB supplementation significantly influenced inflammatory cytokines expression (**Figure 1.4**). Followed by an evaluation on protein level, FRB supplementation could decrease the level of NF-κB p65 and its phosphorylated protein (**Figure 1.5**), muscle atrophy related protein (**Figure 1.6**) and TNF-α (**Figure 1.7**). Other results also showed that FRB supplementation tends to reduce the oxidized proteins and ubiquitinated proteins (**Figure 1.8**).

In short, FRB-supplemented diet was suggested to be able to inhibit the ubiquitin-mediated proteolysis pathway marked by limiting the expression of FBXO32/Atrogin-1 and TRIM63/MuRF1 resulted from the partial inhibition of inflammation in STZ-induced diabetic muscle.

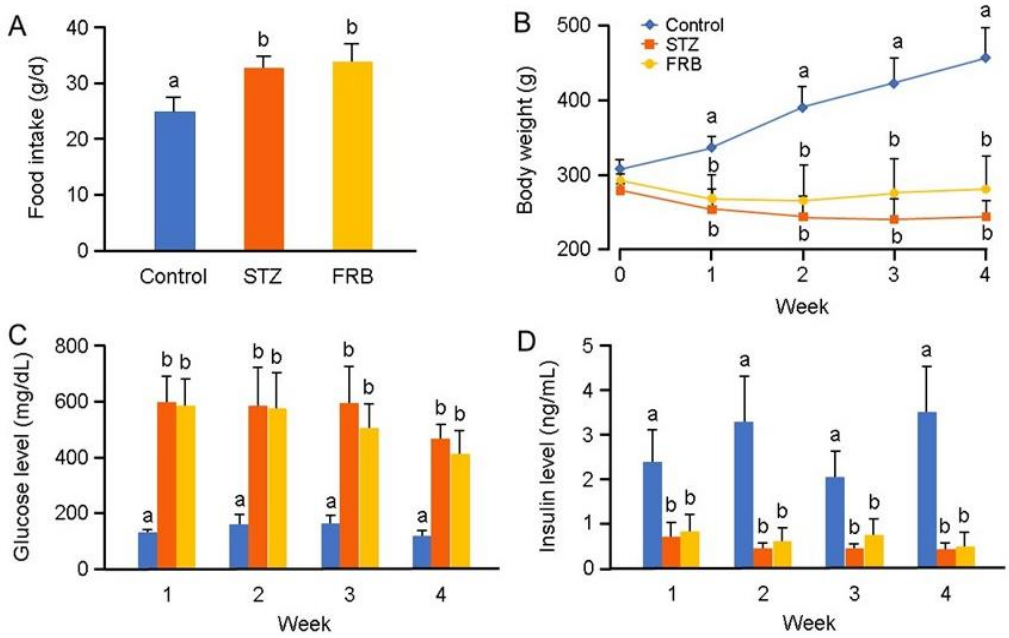

Figure 1.1. General observation during one-month supplementation. (A) The average food intake per day; (B) body weight changes (analyzed using repeated measures ANOVA) (C) Blood glucose level; (D) plasma insulin level. Data are shown as means + SEM. Significance ($p < 0.05$, $n = 5–6$) among groups is denoted by different letters. (Rusbana et al., 2020)

Table 1.1. Blood profile of STZ-induced diabetes after one-month treatment.

Parameter	Unit	Group		
		Control	STZ	FRB
Total protein	g/dL	6.97 ±0.51[a]	5.62±0.38[b]	5.98±0.72[b]
Albumin	g/dL	4.58± 0.35[a]	3.62±0.22[b]	3.68±0.45[b]
Amylase	IU/L	2,308.33± 204[a]	1,045.50 ±326[b]	1,329.00±399[b]
Triglyceride	mg/dL	359.50± 139[a]	69.17±30.2[b]	130.00±101[b]
HDL-cholesterol	mg/dL	33.17± 3.25[a]	43.67±8.66[b]	44.40±10.30[b]
Glucose	mg/dL	153.83± 11.36[a]	680.00±125.65[b]	614.00±68.7[b]
Insulin	ng/mL	4.42±0.99[a]	0.44±0.13[b]	0.51±0.29[b]

Data are shown as the means ± SEM and were analyzed using ANOVA followed by Tukey's *post hoc* test. Significance ($p < 0.05$, $n = 5–6$) among groups is denoted by different letters. (Rusbana et al., 2020)

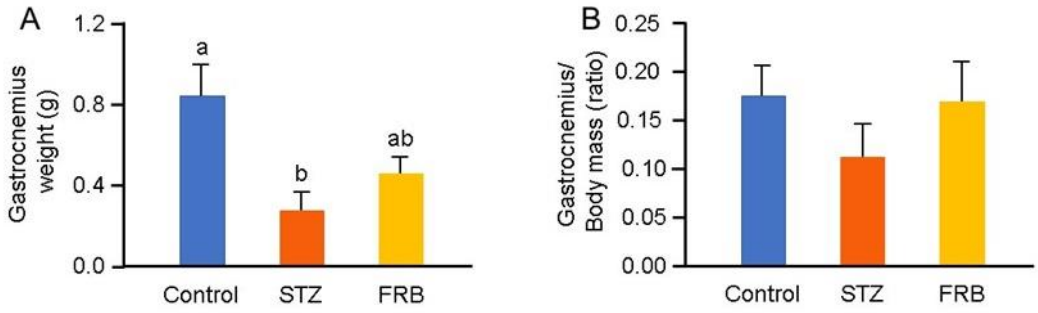

Figure 1.2. FRB supplementation may directly affect muscle condition. (A) Gastrocnemius muscle weight; and (B) Normalized gastrocnemius weight to body mass. Data are shown as the means + SEM. Significance ($p < 0.05$, $n = 5–6$) among groups is denoted by different letters.(Rusbana et al., 2020)

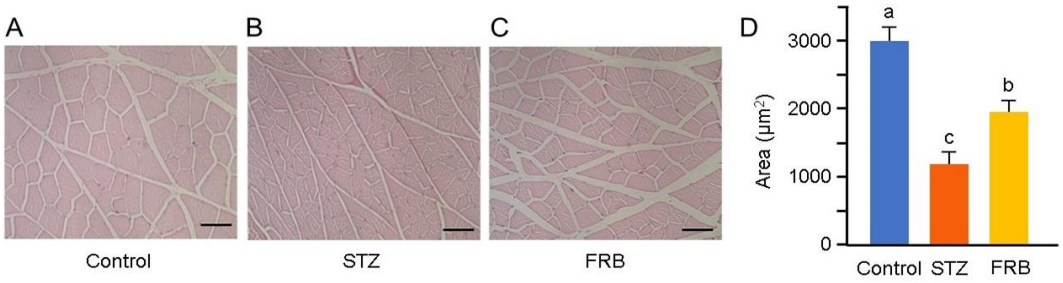

Figure 1.3. FRB supplementation increased the muscle size in rats with STZ-induced diabetes. Representative myocyte cross-sections of the gastrocnemius muscle of the (A) control, (B) STZ, and (C) FRB groups stained with hematoxylin and eosin. (D) The average of the myocyte cross-sectional area. Scale bars represent 50 μm. Significance ($p < 0.05$, $n = 5–6$) among groups is denoted by different letters (Rusbana et al., 2020).

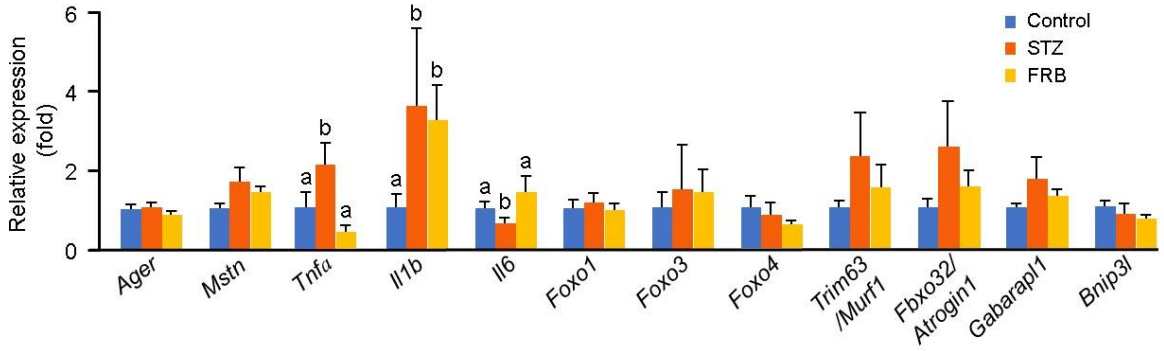

Figure 1.4. FRB supplementation influenced the expression of pro-inflammatory cytokines. Data are shown as the means + SEM. Significance ($p < 0.05$, $n = 5$) among groups is denoted by different letters (Rusbana et al., 2020).

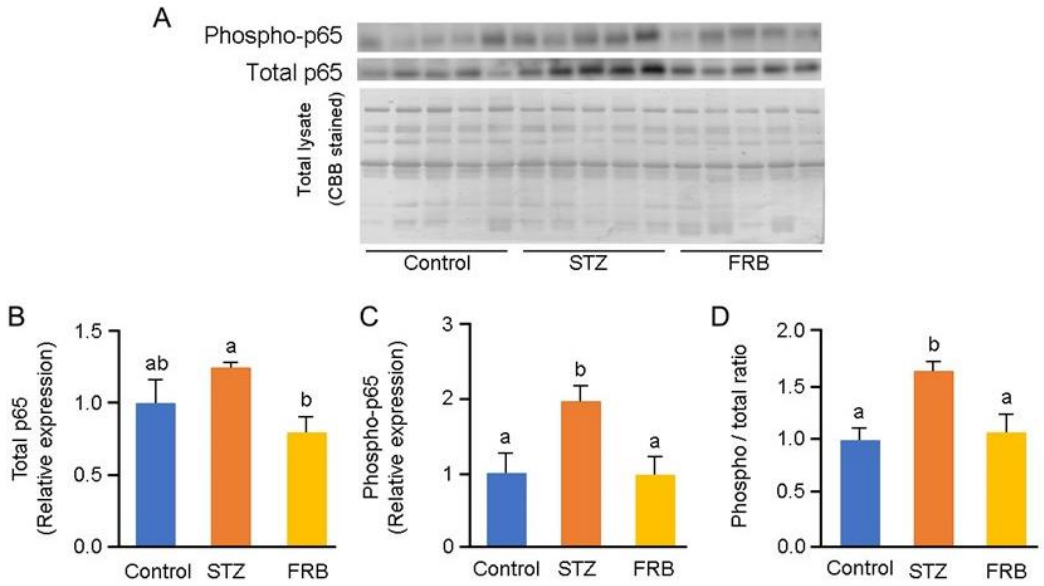

Figure 1.5. FRB inhibits NF-κB activation. Representative blots of (A) phosphorylated p65 (phospho-p65) and total p65, and the quantification of (B) total p65, (C) phospho-p65, and (D) phospho-p65/p65 ratio. Data are shown as the means + SEM. Significance ($p < 0.05$, $n = 5$) among groups is denoted by different letters (Rusbana et al., 2020).

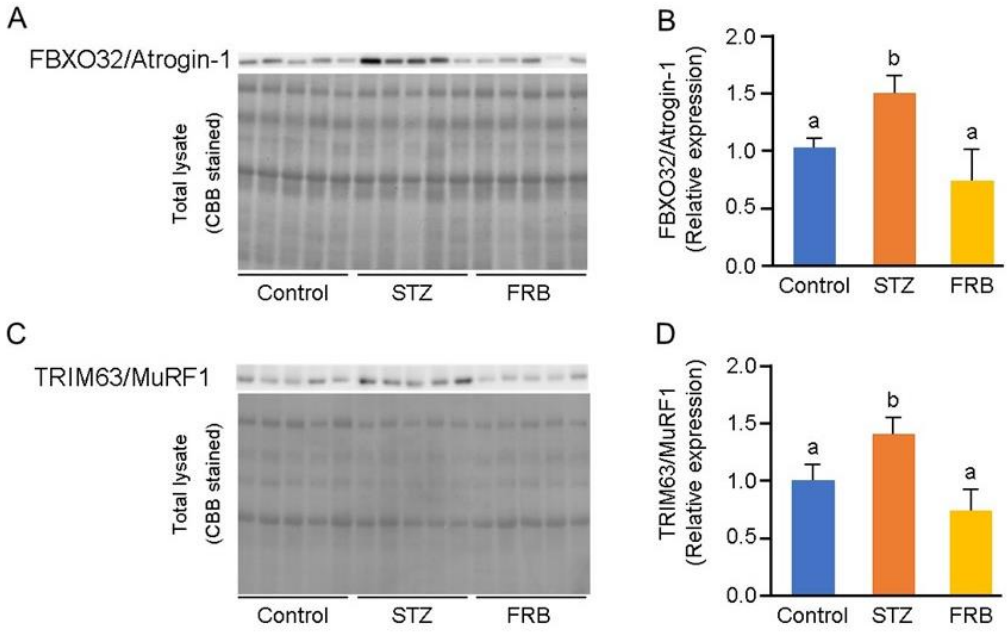

Figure 1.6. FRB decreased the expression of muscle-specific ubiquitin ligases. Representative blots of (A) FBXO32/Atrogin-1 and (C) TRIM63/MuRF1. The quantification of (B) FBXO32/Atrogin-1 and (D) TRIM63/MuRF1. Data are shown as the means + SEM. Significance ($p < 0.05$, $n = 5$) among groups is denoted by different letters (Rusbana et al., 2020).

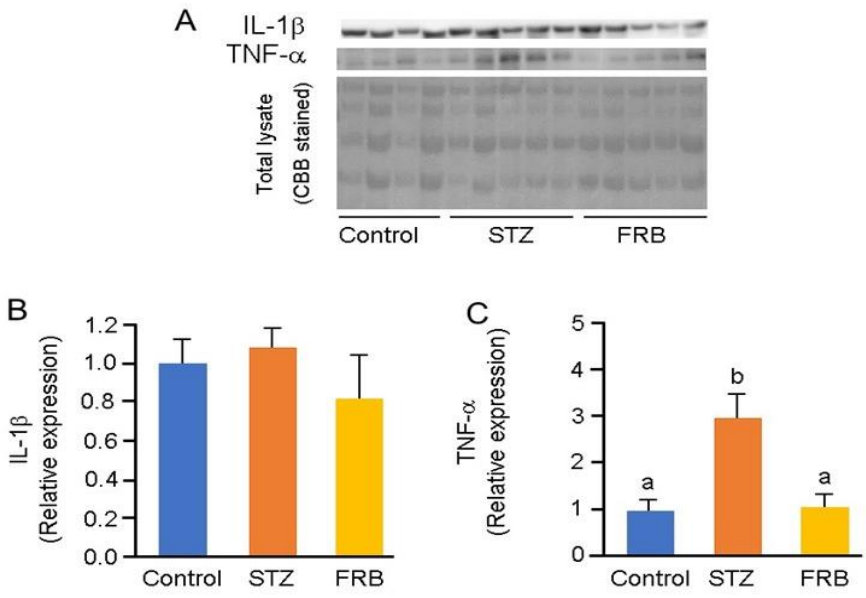

Figure 1.7. FRB decreased the expression of inflammatory cytokine. Representative blots of (A) IL-1β and TNF-α, and quantification of (B) IL-1β and (C) TNF-α. Data are shown as the means + SEM. Significance ($p < 0.05$, $n = 4–5$) among groups is denoted by different letters (Rusbana et al., 2020).

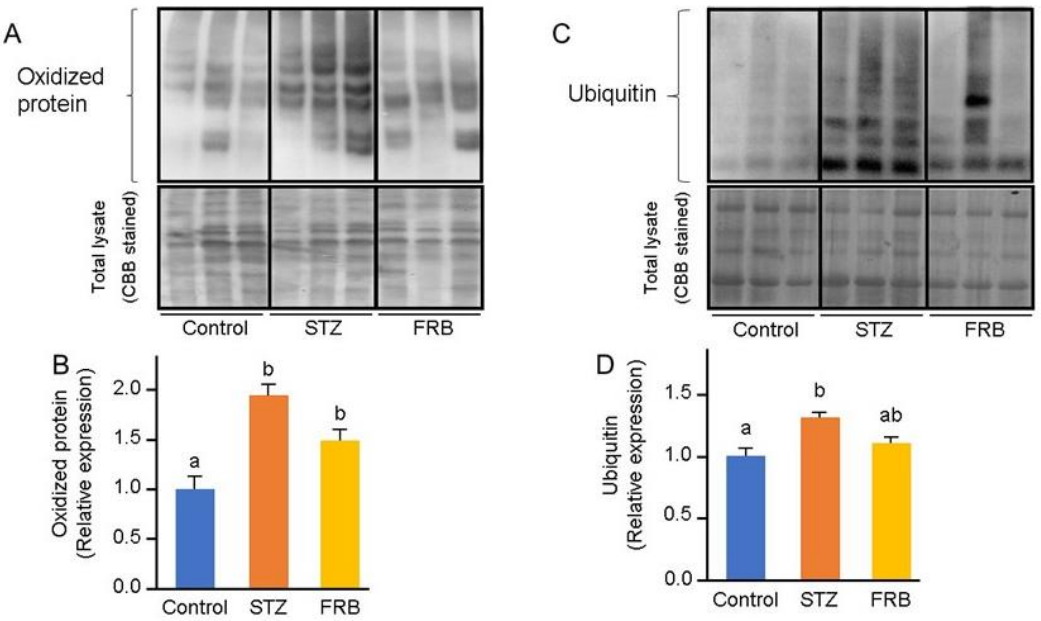

Figure 1.8. The effect of FRB supplementation on the level of oxidized proteins and ubiquitinated proteins. Representative blots of the (A) oxidized proteins and (C) ubiquitinated proteins, and the quantification of the (B) oxidized proteins and (D) ubiquitinated proteins. Data are shown as the means + SEM. Significance ($p<0.05$, $n=5$) among groups is denoted by different letters (Rusbana et al., 2020).

CHAPTER 2

Effect of dietary vitamin K on cognitive impairment in senescence accelerated mouse prone 8 (SAMP8)

Introduction

Nowadays, the increasing evidence showed that VK has a diverse role in the body, beyond blood homeostasis, including the maintenance of bone health (Kuang et al., 2020) and its relation with diabetes mellitus (Booth et al., 2013), the inhibitory function in cardiovascular disease due to its anti-inflammation (Shioi et al., 2020), also the role in another low-grade inflammation diseases. Moreover, clinical studies showed that higher dietary VK intake and its serum level were positively associated with better cognition and behavior among older adults (Alisi et al., 2019; Chouet et al., 2015; Presse et al., 2013b). Interestingly, VK that distribute in the brain is dominated by menaquinone-4 (MK-4) (Harshman et al., 2016). This evidence becomes an interesting object for researchers to explore the relation of VK with brain health and cognitive impairment during aging process.

Providing complete insight into the role of VK at different life stages is time-consuming research. A comprehensive study was done by Carie et al. (2011) using 6, 12, and 20 month age mice which were fed with several levels of phylloquinone in the diet since weaning, resulted in the strengthened concept on the correlation between VK and nervous system by modulation of sphingolipid metabolism (Ferland, 2012). This present study used SAMP8 strain as the brain aging mice model (Akiguchi et al., 2017) to achieve a shorter time of the experiment.

The behaviors changes of SAMP8 as seen as the behavioral alterations observed in aged mice occur in SAMP8 as young as 4 months old (Yanai and Endo, 2016). Brain aging of SAMP8 is induced by the increases in expression of proinflammatory cytokines (Tha et al., 2000) and oxidative stress in the brain (Ota et al., 2012), resulted in neuron loss and gliosis, deposition of amyloid-beta, and accumulation of hyperphosphorylated Tau in the brain, similar to AD patients (Cheng et al., 2014).

Our previous study has shown that VK has an anti-inflammatory effect in the liver of LPS-induced rats (Ohsaki et al., 2006) which might be mediated via the inactivation of the NFκB signaling pathway (Ohsaki et al., 2010). Moreover, we also found that VK inhibits the translocation of NFκB resulted in the attenuates of microglial inflammation (Saputra et al., 2019).

We hypothesized that VK has a specific role in every organ. This is the first long-term study of VK supplementation using SAMP8 which intended to identify the effect of VK on brain aging accomplished by behavioral evaluation.

Material

The components of the AIN-93M standard diet including VK-free vitamin mixture were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) and VK1 from Eisai Co., (Tokyo, Japan). Corn oil (J-Oil Millis, Inc., Tokyo, Japan) was purchased just before every experiment from the local retailer.

Rabbit antibodies against BDNF, CREB, phosphorylated CREB, TNF-α, NFκB p65, and phosphorylated p65 (Cell Signaling Technology, Danvers, MA,

USA) were used for measuring protein expression and all were normalized to the level of beta-actin (Sigma).

Animal and diet

Three-week-old male SAMR1 and SAMP8 mice were purchased from Japan SLC, (Shizuoka, Japan). After 1-week acclimatization, the mice were grouped into 4 group (8 mouse per group) namely SAMR1 (Res), Control (Con), Deficient (Def), and VK supplemented group (Hig).

Diet was made based on the AIN-93M formula. The control and VK-supplemented diets were prepared by adding to the VK-def diet to final concentrations of 0.75 (control) and 75 mg/kg (high supplementation) diet of VK1, respectively. The VK-def diet was prepared by using a VK-free vitamin mixture in the ingredients. The Res and Con groups were fed with a control diet, while the Def group was fed with VK-def diet and high VK1 with high supplementation diet.

The animal use and care protocols were reviewed and approved by the Animal Research-Animal Care Committee of Tohoku University according to the Japanese governmental legislation (2005).

Behavioral test: The Y-maze test

The apparatus that was used for this evaluation was made of black polypropylene walls with 3 arms each 32 cm long, 10 cm wide, and 18cm high. This test started with a familiarization phase followed by test phase that were separated by an intertrial interval (Wolf et al., 2016). In the familiarization phase, one of the three arms of the maze were closed, namely new arm. Each mouse was placed in the maze and was allowed to explore the partially closed maze for 5 min.

In the test phase, all maze`s arms were opened and conducted thirty minutes after the familiarization phase. The animal behavior was video recorded for later analysis. The numbers of arm entries, the time spent in the new arm, and numbers of total arm entries, and the actual alternation were analyzed manually for each mouse over 5 min periods. The actual alternation was defined as entries into all three arms on consecutive choices, such as ABC, CAB, or BCA. The result was expressed as the percentage of alternation identified by the following equation:

Alternation (%) = [(Number of alternations)/ (Total arm entries - 2)] x 100

Behavioral test: The open field test (OFT) and the cued fear conditioning test (FCT)

The OFT and FCT were performed as described previously (Tanemura et al., 2009), with some modification. The experiment was performed in a white-colored wood soundproof box (78 x 63 x 65(H) cm) which was equipped with a light source, a charge-coupled device (CCD) camera, and an audio speaker placed about 50 cm above the center of the box field. The noise tone during the testing was about 50 dB. These tests were performed to evaluate the behavior in the one years old of mice.

The OFT was performed to measure locomotor and emotional activity. The test was done on the white plastic box (50 x 50 x 30 (H) cm) with the lighting system was set 25 lux at the center of the field together with a CCD camera to record the movement of the mouse. Behavior parameters were measured using Image OF2 software (O`Hara & Co. Ltd., Japan).

The FCT was performed to evaluate the learning and memorize ability based on the freezing response after conditioning cued treatment. The conditioning apparatus consists of the clear plastic chamber (17 x10 x 10 (H) cm) with the inner wall of the chamber was covered with black and white plastics stripes, while the floor equipped with stainless steel rods (2 mm diameter) spaced 5 mm apart for electric foot shock to the mouse. The light was set 50 lux at the center of the floor and a CCD camera was used to record the mouse movement. Forty seconds after it was placed in the chamber, the mouse was treated with six tone-shock pairings (20 s of tone at 65 dB, directly followed by 2 s of 0.07 mA electric shock in the 19^{th} until 20^{th} second of the tone-shock pairings cycle time) per 60 s. After 6 minutes of conditioning treatment, the mouse was then returned to the home cage.

One day after the conditioning treatment, as the cued fear test, the mouse was placed in a novel chamber (lacking black-white plastic stripes and stainless still rods). After 3 min, the conditioning tone (without foot-shock) was presented for 3 min. The freezing responses of the mouse were measured by Image FZ2 software as a consecutive 2 s period of immobility. The freezing rate (%) was calculated as:

Freezing rate (%) = (Freezing time / total session time) x 100

Western blotting

The level of BDNF, CREB, phosphorylated CREB, TNF-α, NFκB p65, and phosphorylated p65 protein in hippocampus were analysed by western blotting referred to Rusbana et al. (2020).

Statistical analysis

Data are presented as the mean + SEM. Statistical analysis was performed using SigmaPlot version 12.5 (San Jose, CA, USA). The Tukey's test was used to analyze the comparisons among the groups. All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($p < 0.05$).

Result and discussion

Food intake per day of the mouse for both SAMR1 and SAMP8 is 4.5 – 4.6 g on average and it is not different for all groups (**Figure 2.1A**). The bodyweight of SAMR1 significantly higher than SAMP8 groups (**Figure 2.1B**). This result indicates that the concentration of VK in the diet does not affect the food intake level. After 12 months of the supplementation, the mice were euthanized and the concentration of MK-4 in the cortex was measured (**Figure 2.2**). The concentration of MK-4 in the cortex of the high supplemented group was significantly higher compared to the other groups.

Regarding the Y-maze test, at 4 months of age, there is no difference among the groups in the alternation, frequency, and time spent in visiting the novel arm (**Figure 2.3A**), while at 8 months of age (**Figure 2.3B**), the deficient group showed the lowest performance on alternation and spent time on the novel arm. Behavior evaluation using an open field test at 12 months of age showed different results among all groups (**Figure 2.4A and B**), also on the cued fear conditioning test (**Figure 2.4.C and D**) showed that the deficient group has the lowest response on cued.

Hypothesis that BDNF is take a role in this study, further we investigate the level of this protein. The result showed that total BDNF of the high supplemented diet group was higher compared to other groups (**Figure 2.5A and B**). The investigation on PKA, ERK, and CREB expression showed that VK supplementation enhanced the activation of ERK (**Figure 2.6 A and B**).

To confirm the anti-inflammatory effect of the VK supplemented diet on the brain, we measured the level of total NF-κB p65 and its phosphorylated form, and TNF-α in the hippocampus (**Figure 2.7A-D**). We found that the level of NF-κB was not different among all groups, while TNF-α tended to be lower in the high supplemented group.

Briefly, deficient VK diet accelerate the cognitive impairment in SAMP8 mouse and supplementation VK may influence the BDNF production with unclear effect on maintaining brain condition until 12 months intervention.

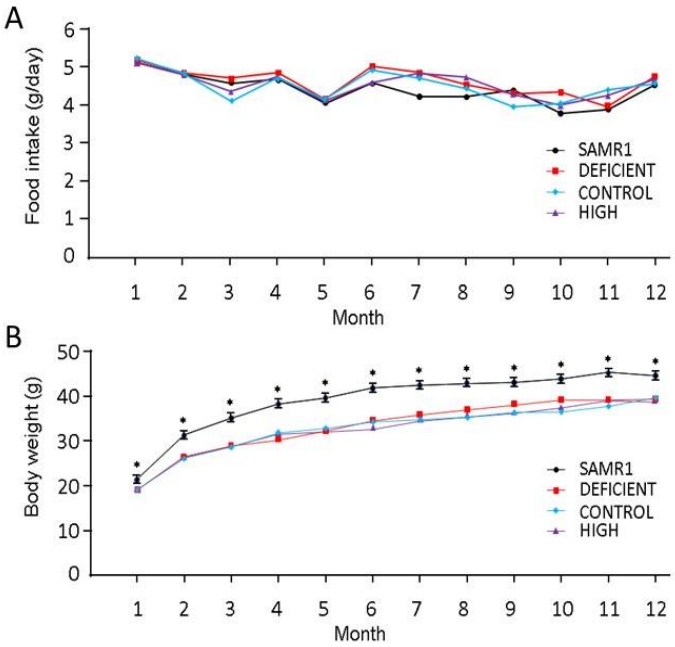

Figure 2.1. General observation during 12-month supplementation. (A) Food intake; (B) Body weight. Data are shown as the means + SEM. Significance among groups (Tukey's post hoc test, $p < 0.05$, $n = 4$) is denoted by asterix.

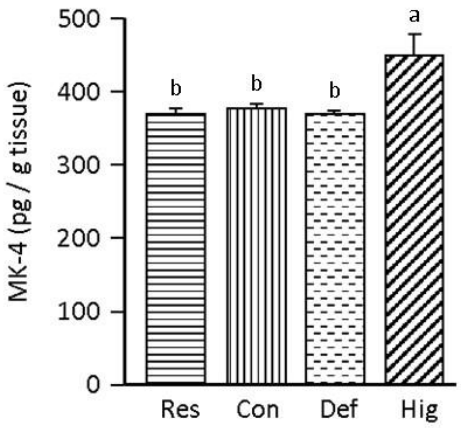

Figure 2.2. The concentration of MK-4 in the brain cortex. Data are shown as the means + SEM. Significance (Tukey's post hoc test, $p < 0.05$, $n = 4$) among groups is denoted by different letters.

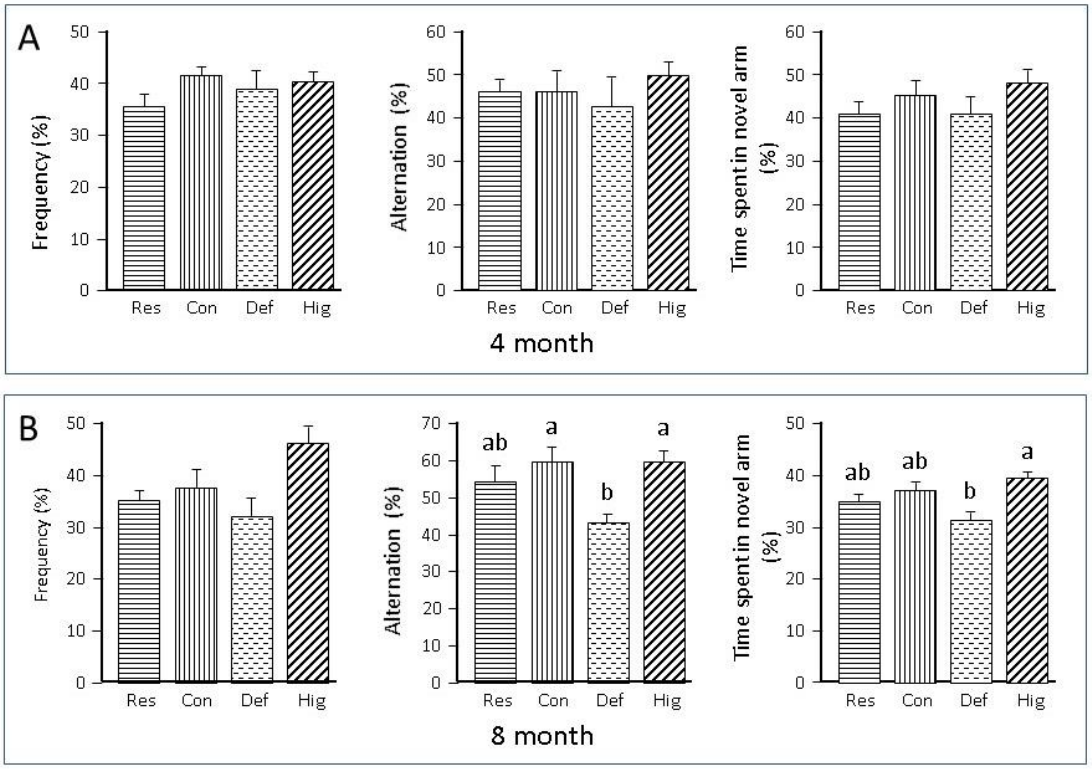

Figure 2.3. Deficient VK diet accelerated cognitive impairment. Y-maze test result on (A) 4 month and (B) 8 months. Data are shown as the means + SEM. Significance (Tukey's post hoc test, $p < 0.05$, $n = 4$) among groups is denoted by different letters.

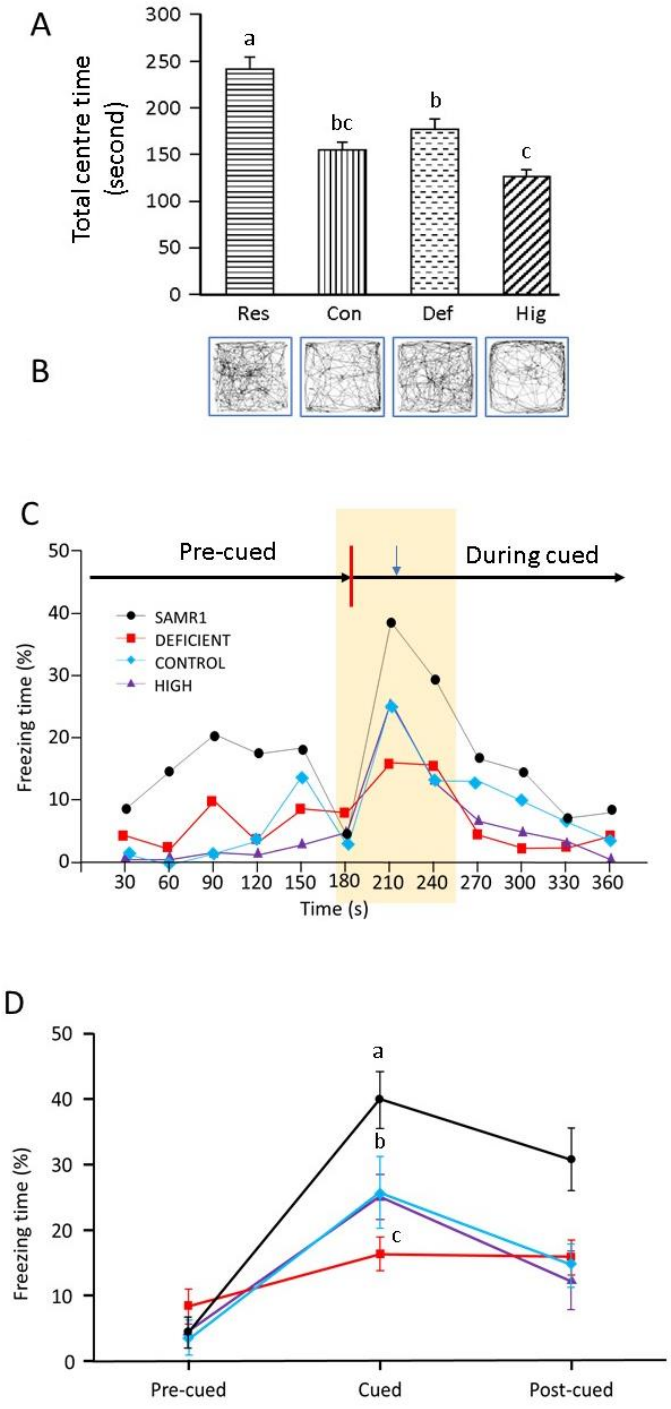

Figure 2.4. High VK supplementation diet functioned as neuroprotective agent. Open field test result: (A) Total centre time and (B) representative track movement of the mice; cued fear conditioning test result: (C) overall freezing time and (D) cued initial response. Data are shown as the means + SEM. Significance (Tukey's post hoc test, $p < 0.05$, $n = 4$) among groups is denoted by different letters.

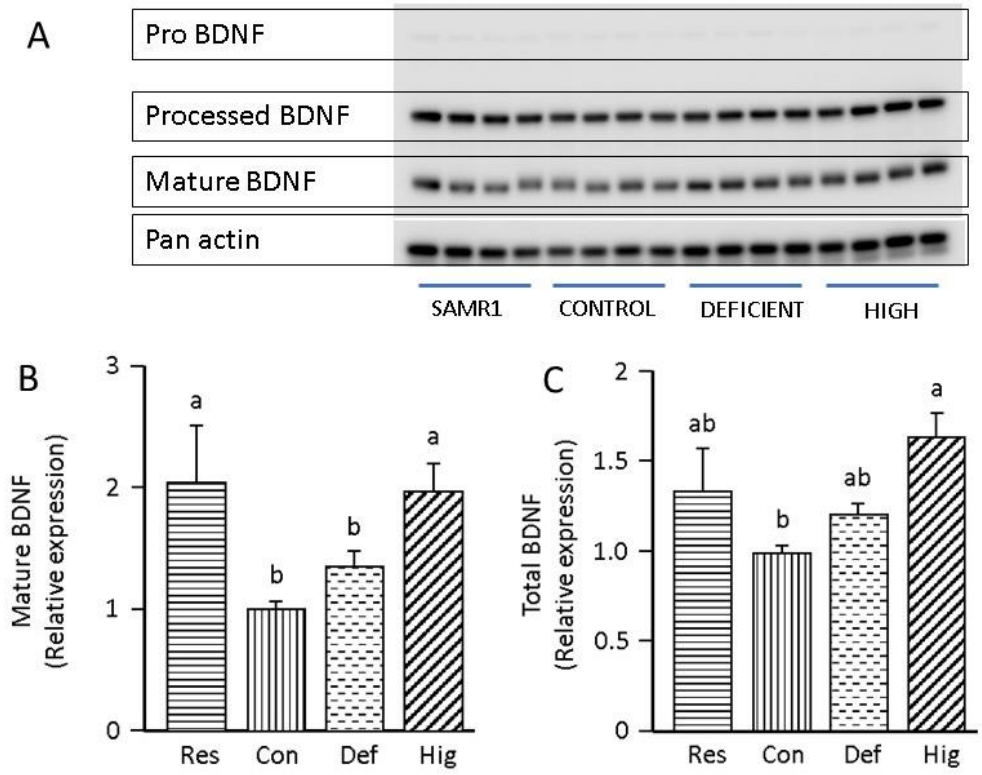

Figure 2.5. High VK supplementation diet increased total BDNF level. Representative blots of (A) BDNF fraction and the quantification of (B) Total and (C) mature BDNF. Data are shown as the means + SEM. Significance (Tukey's post hoc test, $p < 0.05$, $n = 4$) among groups is denoted by different letters.

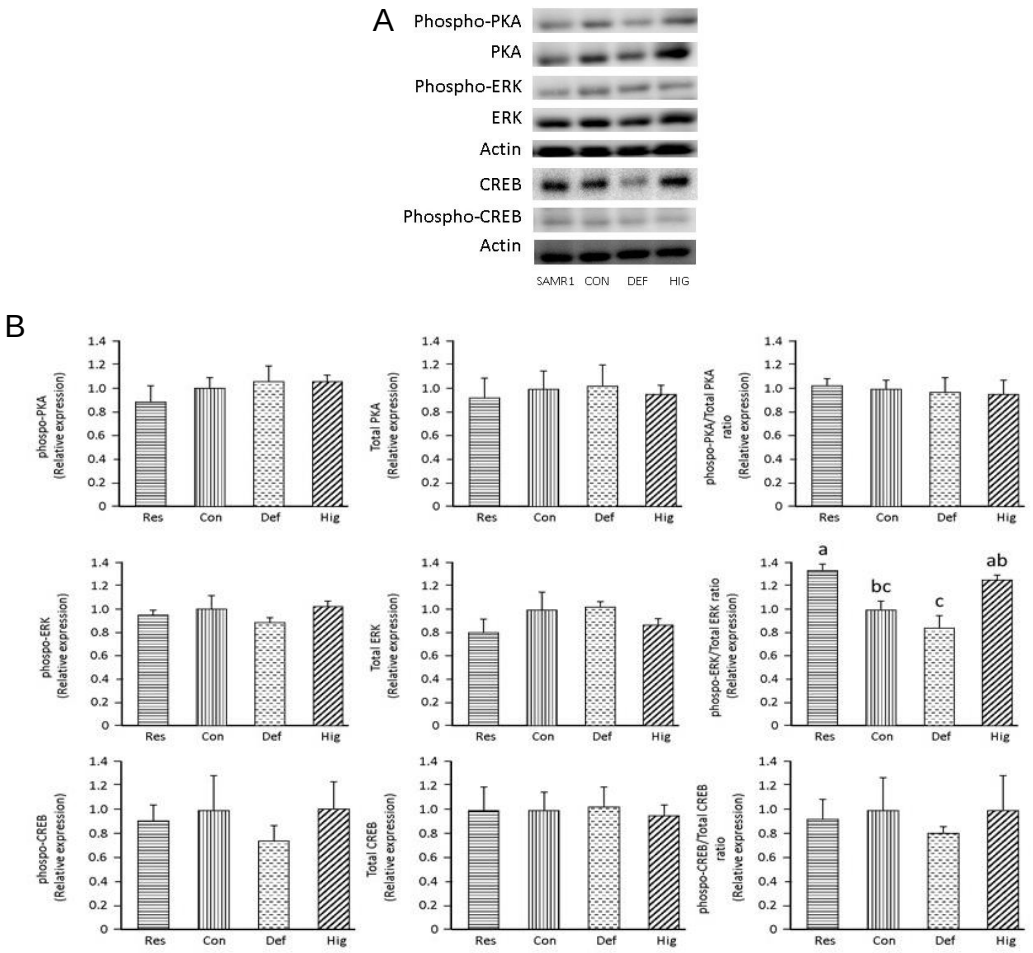

Figure 2.6. High VK supplementation influenced ERK activation. Representative blots of (A) PKA, ERK, and CREB and its phosphorylation protein; and the quantification of (B) PKA, ERK, and CREB and its phosphorylation and total protein ratio relative expression. Data are shown as the means + SEM. Significance (Tukey's post hoc test, $p < 0.05$, $n = 4$) among groups is denoted by different letters.

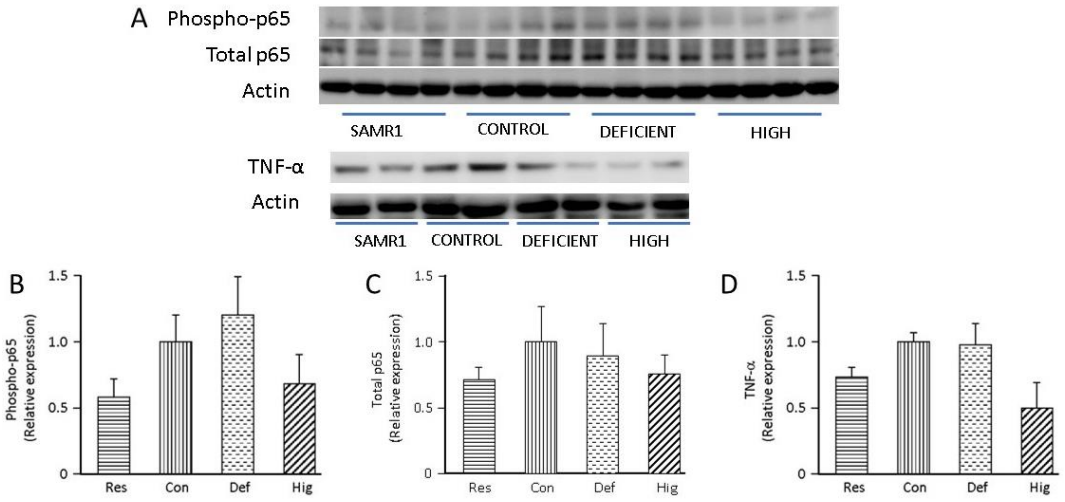

Figure 2.7. High VK supplementation influenced pro-inflammatory cytokines expression. Representative blots of (A) total NF-κB p65, phospho-p65, and TNF-α; and the quantification of (B) phospho-65; (C) total p65; (D) TNF-α relative expression. Data are shown as the means + SEM. Significance (Tukey's post hoc test, $p < 0.05$, $n = 4$) among groups is denoted by different letters.

CHAPTER 3

Discussion and conclusion

Discussion

Aging condition corelates with oxidative stress which leads to the frailty in muscular and cognitive function due to the chronic inflammation effect. Using two representative models, we evaluated the function of FRB and VK in facing the muscle atrophy and cognitive impairment in aging animal model.

Firstly, we reported here that the STZ-induced diabetic rat model has been represented the type 1 diabetes symptoms such as hyperglycemia, hypoinsulinemia, hyperurination, polyphagia and followed by muscle atrophy and weight loss (Wang-Fischer and Garyantes, 2018). In this study, we showed that injection of 40 mg of STZ per kg body weight in our animal model resulted in the muscle atrophy, which was characterized by polyphagia and resulted in decreased plasma insulin levels and elevated blood glucose levels, followed by a reduction in the body weight (Figure 1.1). Moreover, similar to the studies of Rai et al.(2016), the blood profile (Table 1.1) showed that the damage of the pancreatic β cells by STZ results in the hyperglycemic condition caused oxidative stress and altered the complication such as hepato-renal inflections and lipid mobilization.

Diabetes mellitus could enhanced the protein degradation by the activation of ubiquitin-proteasome system leads to muscle atrophy (Roy, 2013b; Wang et al., 2006), and also could resulted on the removing contractile proteins and organelles caused the shrinkage of muscle mass and myofiber size (Bonaldo and Sandri, 2013;

Kalyani et al., 2014). In this study, the diabetic complications represented by the deflation of bodyweight, which was followed by shrinkage of muscle fibers in the diabetic groups marked by the different weights of the gastrocnemius muscle (Figure 1.2). Even though FRB supplementation did not prevent body weight loss, one month of FRB supplementation partially reversed the loss of gastrocnemius muscle mass and ameliorated the muscle shrinkage (Figure 1.3).

Hyperglycemic conditions induce the diabetes-related genes coding for cell surface stimulators which contribute to the generation of oxidative stress in diabetic organs. It has been shown that Ager -the regulator of glucose homeostasis- and Mstn -the regulator of myogenesis- are highly expressed in diabetic conditions (Coleman et al., 2016; Pinto-Junior et al., 2018), whereas Tnfa is highly expressed in hypoinsulinemia (Federici et al., 2005). In this study, we showed that FRB supplementation significantly reduced Tnfa mRNA expression (Figure 1.4).

Studies have shown that the hyperglycemic conditions could activates NF-κB by oxidative stress which directly increases the activity of the ubiquitin-proteasome proteolytic pathway in muscles (Eley and Tisdale, 2007; Roy, 2013) The hyperglycemic and hyperinsulinemia in the diabetic muscle also triggered FOXO family proteins activation which regulate FBXO32/Atrogin-1 and TRIM63/MuRF1 (Sandri et al., 2004), and dependently regulate BNIP3L and GABARAPL1 (O'Neill et al., 2019). The result of our study showed that FRB supplementation was also shown to inhibit the activation NF-κB (Figure 1.5) and resulted in the decrease of both FBXO32/Atrogin-1 and TRIM63/MuRF1 level (Figure 1.6).

TNF-α and IL-1β which expressed via NF-κB pathway, could be a positive feedback loop that enhances the activation of NF-κB itself, resulted in the intensify the muscle degradation (Li et al., 2008). Moreover, TNF-α treatment in C2C12 cells was proven to overexpressed FBXO32/Atrogin-1 (Yuan et al., 2015). Besides that, TNF-α induced NF-κB activation also found to increase the level of TRIM63/MuRF1 (Cai et al., 2004). This current study showed that FRB could decreased the TNF-α protein levels (Figure 1.7) and the tendency to reduce the oxidized proteins and ubiquitinated proteins (Figure 1.8) confirmed that this supplementation will consequently inhibit the ubiquitin-proteolysis pathways.

In short, FRB-supplemented diet was suggested to be able to inhibit the ubiquitin-mediated proteolysis pathway, thereby limiting the expression of FBXO32/Atrogin-1 and TRIM63/MuRF1. Thus, the reduced expression of these specific protein levels in the FRB group compared to that in the STZ group confirmed the beneficial effects of FRB supplementation in regulating the muscle atrophy in diabetic circumstance. Therefore, further studies are needed to elaborate the role of FRB components on preventing muscle atrophy during aging, diabetes, or other chronic diseases.

On the second theme, we investigate the possibility of VK supplementation on prevent the cognitive impairment using SAMP8 model. On this study we showed that the concentration of VK in the diet does not affect the food intake level of the mouse. The food intake for both SAMR1 and SAMP8 is not different for all groups (**Figure 2.1A**). However, the bodyweight of SAMR1 significantly higher than SAMP8 groups (**Figure 2.1B**). This result indicates that the same amount of food intake followed by the difference of body weight for both

control and treatment group between SAMR1 and SAMP8, which also shown in other studies (Jeng, 2009), confirms that the food efficiency of SAMP8 is lower compare to SAMR1.

The concentration of MK-4 in the brain cortex of the high VK diet group was significantly higher compared to the other groups (**Figure 2.2**). This result is similar on the study of long-term VK supplementation in rats which was showed that 12-month supplementation of VK resulted in a significant difference in MK-4 concentration in the brain but not significant after 22-month supplementation (Ferland et al., 2016). This evidence suggests that advancing the age or acceleration of senescence causes the lowering accumulation of MK-4 in the brain region and supplementation could retain the decrease.

The Y-maze test at 4 months of age did not showed the difference among the groups (**Figure 2.3A**), while at 8 months of age (**Figure 2.3B**) the deficient VK diet group showed the lowest performance on alternation and spent time on the novel arm. This result indicates that the deficient VK diet group experienced cognitive impairment faster than the other groups. Even though the behavioral alterations occur in SAMP8 as young as 4 months old (Yanai and Endo, 2016), the Y-maze test in this study could detected the change in 8 months of age.

The open field test at 12 months of age exhibited the different results among all groups (**Figure 2.4A and B**). Comparing among the groups of SAMP8, this evaluation result indicates that the high VK diet group has the least amount of total center time suggest that the high supplementation diet might be beneficial for keeping the health of the aged brain. Moreover, the cued fear conditioning test

(**Figure 2.4.C and D**) also showed that the deficient VK diet group has the lowest response on cued which indicates that a deficient diet influences the cognitive impairment of the aged brain.

BDNF is the most abundant growth factor in the brain, which plays an important role in sustaining physiological processes of the brain and promotes neuroprotection and neuro-regeneration (Palasz et al., 2020). In this study, the total BDNF of the high VK diet group was higher compared to other groups (**Figure 2.5A and B**). While the hypothesis that VK enhance PKA and CREB activation still unclear in this study, our result showed that VK supplementation enhanced the activation of ERK (**Figure 2.6 A and B**). From this result we could indicates that the functional effect of VK on healthy brain and behavior performances is related to BDNF level in the brain. Even though the mechanism on how high VK supplementation diet on total BDNF on SAMP8 mouse in this study still need more investigation, but another study in our lab showed that supplementation of VK activates the PKA-CREB-BDNF pathway. Another possibility on how VK have the indirect effect on BDNF production is that VK regulates the level of sphingolipids (Carrié et al., 2004) in which one of the sphingolipids (ganglioside GQ1b) could enhance the production of BDNF (Shin et al., 2014) and resulted in the improvement of cognitive impairment in the animal model (Jung et al., 2008; Shin et al., 2019).

Evaluating the anti-inflammatory effect of the VK supplemented diet on the brain, we measured the level of total NF-κB p65 and its phosphorylated form, also TNF-α in the hippocampus (**Figure 2.7A-D**). The result showed that the level

of NF-κB was not different among all groups, while TNF-α tended to be lower in the high VK diet group. This result indicated that a high VK diet influenced the expression of pro-inflammatory cytokine directly similar in the previous study (Ohsaki et al., 2010; Ohsaki et al., 2006) or indirectly via BDNF inhibition effect (Han et al., 2019).

The general result of the behavioral test on SAMP8 related to VK supplementation diet showed that deficient diet or insufficient amount of VK daily intake could accelerate the cognitive impairment, while the effect of high supplementation of VK still could not elucidated after 12 months of maintenance. The sufficient intake of VK maybe correlated with BDNF expression which has crucial function on brain maintenance.

Conclusion

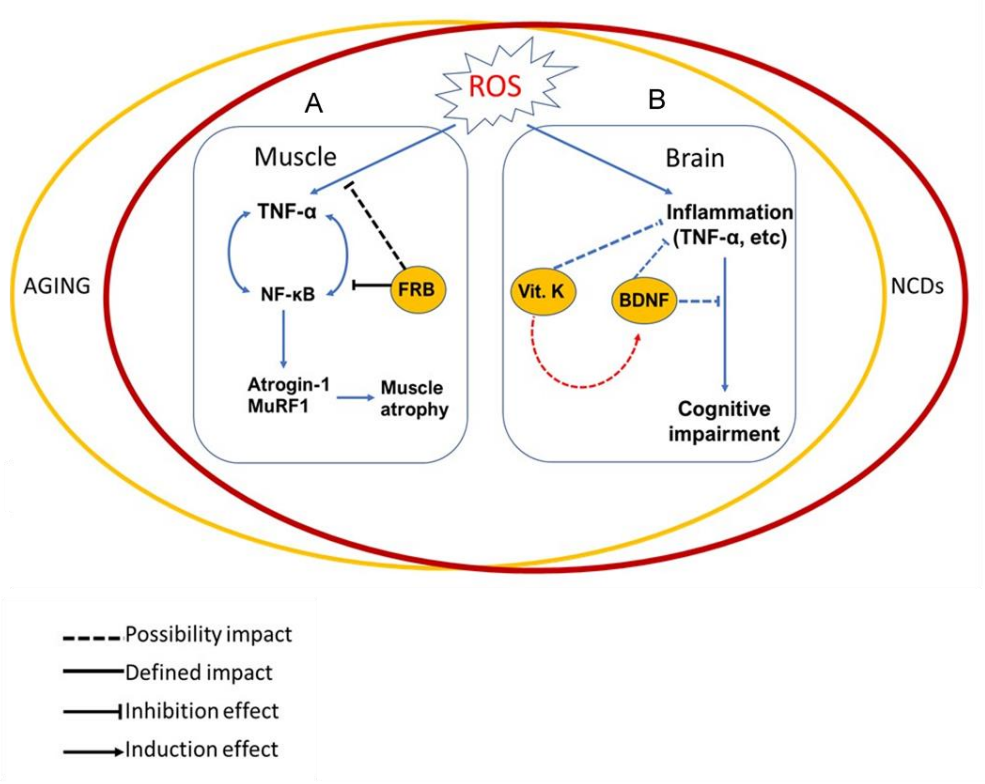

Figure 3. The propose mechanism of FRB and VK on ameliorate the aging effects.

High levels of pro-inflammatory markers in the blood and other tissues are often detected in older individuals and predict the risk frailty, multimorbidity, and decline of physical and cognitive function (Ferrucci and Fabbri, 2018). SAMP8 as an ageing model also shows a high level of inflammatory cytokine especially TNF-α (Xu et al., 2020) as well as in the streptozotocin-induce diabetes model (Siqueira et al., 2010). In this study, both VK and FRB showed the potency as an anti-inflammatory agent on reducing the level of TNF-α, which partially effect on managing the muscle atrophy in a diabetic muscle rat model and withstanding the brain condition on the SAMP8 ageing model (**Figure 3**.).

The decreased of ROS-induced TNF-α level on STZ-induced diabetic muscle by FRB supplementation effects on inhibition of TNF- α positive feedback loop and the deflation of NF-κB activation. In addition to having the capacity to decrease the TNF-α protein levels, FRB supplementation was also shown the tendency on reducing the oxidized proteins. This process was further confirmed that FRB supplementation reduced the activation of the NF-κB pathway that occurred owing to STZ-induced diabetes and consequently inhibit the production of FBXO32/Atrogin-1 and TRIM63/MuRF1 resulted in the increase of myofiber cross-sectional area (**Figure 3A**).

VK supplementation may partially support on neuroprotective effect on the aged brain in SAMP8 while a deficient diet of VK may accelerate cognitive impairment. High supplemented VK diet tented to decrease the inflammatory cytokine due to the direct effect of VK or indirectly by the anti-inflammation effect of BDNF in which the level of BDNF is induced by VK supplementation (**Figure 3B**).

In general, FRB and VK were found to have a partial functional effect on the development of healthy aging in age-related animal models. The anti-inflammatory effect of these substances is the main function in the role of managing muscle atrophy and cognitive impairment. Supplementation of these substances for the human diet needs further evaluation.

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LIST OF FIGURES

LIST OF TABLES